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Monoclonal Antibodies Directed Against the Epstein-Barr Virus-Encoded Nuclear Antigen 1 (EBNA1): Immunohistologic Detection of EBNA1 in the Malignant Cells of Hodgkin's Disease

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Monoclonal antibodies directed against the Epstein-Barr virus nuclear protein 1 (EBNA1) were used to examine conventional paraffin sections from a series of EBV-associated lymphoproliferative disorders by immunohistochemistry. The presence of latent EBV infection in tumor cells was determined by in situ hybridization for the Epstein-Barr virus early RNAs (EBERs). Of those EBER-positive cases a total of 28 of 40 cases of Hodgkin's disease, 3 of 3 cases of Burkitt's lymphoma, and 8 of 8 cases of human immunodeficiency virus-associated cerebral B-cell lymphoma expressed detect-

able amounts of EBNA1. In the positive cases, expression was confined to the tumor cells. No reactivity was detected in EBV-negative cases of the above tumors or in 8 cases of EBV-negative cases of large cell anaplastic non-Hodgkin lymphoma. This report provides the first unequivocal evidence for the expression of the EBNA1 protein in the tumor cells of Hodgkin's disease and validates an important reagent with which to analyze the role of EBV in various virus-associated malignancies.

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THE EPSTEIN-BARR VIRUS (EBV) is a human herpesvirus that infects approximately 90% of the world's adult population.¹ Whereas primary EBV infection in early life is usually asymptomatic, infection in early adulthood is associated with a self-limiting lymphoproliferative disease, infectious mononucleosis.¹ Once infected, individuals carry the virus as a life-long infection with persistence of EBV in the lymphoid compartment.² Although the majority of virus-infected individuals remain healthy, EBV is strongly implicated in the pathogenesis of African Burkitt's lymphoma (BL)³ and nasopharyngeal carcinoma (NPC).^{4,5} EBV is also associated with lymphoproliferative disease in immunosuppressed individuals, where it is found in virtually all post-transplant lymphomas and some acquired immunodeficiency syndrome-associated lymphomas, particularly of the central nervous system.⁶

Over the last few years evidence has been accumulating that implicates EBV in the etiology of another malignancy Hodgkin's disease (HD), a tumor of the lymphoreticular system. The malignant population of Hodgkin and Reed-Sternberg (HRS) cells found in HD lymph nodes are unusual in that they form only a minority of the tumor mass and are of uncertain cell lineage.⁷ Seroepidemiological studies have shown elevated antibody titers to EBV antigens in HD patients at the time of presentation as well as in serum samples

taken some years before the onset of the disease.^{8,9} There is also a threefold increased risk of developing HD after infectious mononucleosis.¹⁰ Recently, more direct evidence linking EBV with HD has been published. Thus, EBV genomes have been detected in tumor material in 19% to 50% of HD cases and in situ hybridization has localized these viral genomes to the malignant HRS cells.¹¹⁻¹³ The demonstration of monoclonal EBV episomes in HD suggests that clonal cellular proliferation is initiated subsequent to EBV infection.¹³ Expression of the small EBV-encoded nonpolyadenylated RNAs EBER1 and EBER2 in HRS cells has been reported and is consistent with the finding of these abundant transcripts in all situations where latent EBV infection is sustained.¹⁴ An etiological role for EBV in the pathogenesis of HD is further supported by the finding that the oncogenic latent membrane protein 1 (LMP1) viral protein is highly expressed in the malignant HRS cells of EBV-positive HD cases in the absence of EBNA2 expression.^{15,16} This restricted pattern of EBV latent protein expression is similar to that found in NPC.^{17,18} In particular, analysis of EBV transcription in fresh HD biopsy specimens identified EBV nuclear protein 1 (EBNA1) mRNA initiating from the *Bam*HI F region of the viral genome (so-called F promoter) as in NPC and BL, rather than the Cp and Wp promoters that are used to direct transcription of all the EBNA1s in lymphoblastoid cell lines (LCLs).¹⁹⁻²¹ EBNA1 is consistently expressed in all EBV infections and appears to be essential for viral DNA replication and the maintenance of viral episomes in the infected cell.²² It has been inferred from the RNA data that EBNA1 is expressed at the protein level in HD. However, the lack of suitable monoclonal antibody (MoAb) reagents has prevented the direct demonstration of this protein in HRS cells. In this report, we describe the generation of EBNA1-specific MoAbs and their use to detect EBNA1 in conventional paraffin tissue sections.

MATERIALS AND METHODS

Cell cultures and virus. A range of LCLs carrying different EBV isolates and EBV-positive and EBV-negative BL cell lines were maintained in RPMI 1640 as described.²³⁻²⁷ The propagation of recombinant baculovirus expressing EBNA1 in SF158 cells was as described.²⁸

Production of MoAbs. The antigen, purified from *Escherichia*

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coli as a lambda-cro/lacZ-EBNA1-fusion protein was supplied by Bill Sugden (Madison, WI).²⁹ Approximately 20 µg of antigen suspended in phosphate-buffered saline (PBS) was emulsified with complete Freund's adjuvant and injected subcutaneously and intraperitoneally into Lou/c rats. Fusion of the myeloma cell line P3X63 Ag 8-653 with the rat immune spleen cells was performed according to the general procedure.³⁰ Hybridoma supernatants were tested in a solid-phase immunoassay using cell extract derived from SF158 insect cells infected with either recombinant EBNA1-expressing baculovirus or wild-type baculovirus as a control. Extracts were prepared by sonication of infected cells that were suspended in PBS. The cell lysate was cleared by high-speed centrifugation and used to coat polystyrene microtiter plates. Culture supernatants were incubated for 1 hour, and bound rat MoAbs were detected with goat-antirat IgG coupled with horseradish peroxidase, using O-phenylenediamine as substrate. The Ig subclass was determined in a solid-phase enzyme-linked immunosorbent assay (ELISA) with mouse antirat antibodies for capture and biotinylated monoclonal mouse antirat Ig heavy chain-specific antibodies (IgM and IgG subclasses) as indicators.³¹

The antibodies that reacted positively were confirmed by immunofluorescence using SF158 cells infected with the EBNA1-expressing baculovirus and by Western blotting using various EBV-positive and EBV-negative cell lines.^{26,28} Antibodies that yielded more than one band in the Western blot or that reacted with proteins in EBV-negative cells were not considered further. Clones designated EBNA1-1H4-1 (IgG2a) and EBNA1-2B4-1 (IgG1) were subsequently tested by immunoprecipitation analysis, because they reacted strongly with a variety of EBNA1 proteins from different cell lines in the immunoblot. The preparation of cell extract, immunoprecipitation, and subsequent detection of precipitated EBNA1 was performed essentially as described earlier.²⁶ The nonspecific rat IgG1 MoAb Rmt6 (E. Kremmer, unpublished) served as a negative control.

Specimens. Tumor samples were obtained as either formalin-fixed paraffin wax blocks or frozen tissue from the Queen Elizabeth Hospital (Birmingham, UK) and from the Institute of Pathology (Homburg, Germany). Paraffin wax sections were cut at 5 µm and attached to glass slides by incubation overnight at 60°C. Slides had been pretreated with an adhesive (Frotissuer; The Binding Site, Birmingham, UK). Frozen sections were prepared at 6 µm on a cryostat and then fixed in 4% paraformaldehyde for 20 minutes. Cytospin preparations were made from cell lines and fixed in 4% paraformaldehyde for 20 minutes before immunostaining. Selected lines were pelleted, processed to paraffin wax, and sectioned as described above. In addition, B95-8 LCLs and Louckes lines were used to generate tumors in SCID mice as previously described.³² Tumors obtained in this way were either snap-frozen or processed to paraffin wax.

Immunofluorescent staining. Cell cytospin preparations or frozen tissue sections were fixed as described above and either directly analyzed or pretreated in 0.1 mol/L citrate buffer, pH 6.0, for 50 minutes in a microwave oven. The slides were then incubated with MoAbs 1H4-1 or 2B4-1 diluted 1:50 or 1:100 in PBS containing 20% normal rabbit serum; the PBS/20% normal rabbit serum solution was used for all subsequent dilutions. After a 2 hours of incubation at 37°C, the slides were washed in PBS and then incubated with a 1:50 dilution of fluorescein isothiocyanate (FITC)-conjugated goat antirat Ig (Sigma, St Louis, MO) for 1 hour at 37°C. At this stage the slides were either (1) washed in DABCO solution (90% glycerol, 10% PBS, and 2.5% w/v 1,4-diazabicyclo (2,2,2)octane, pH 8.6) and viewed under a UV-fluorescence microscope or (2) treated to a second round of staining using a 1:50 dilution of fluorescein isothiocyanate-conjugated rabbit anti-goat Ig (Sigma) before mounting and viewing.

Immunohistology. Initially, specimens were analyzed for the

presence of latent EBV infection by in situ hybridization using digoxigenin-labeled riboprobes to detect the EBER1 and EBER2 transcripts according to published procedures.^{33,34} The presence of latent EBV infection was confirmed on selected cases by immunohistochemistry for LMP1 using the CS1-4 MoAb as described previously.³⁵

Immunohistochemistry for EBNA1 was then performed on each specimen. Briefly, paraffin sections were deparaffinized and pretreated in 0.1 mol/L citrate buffer, pH 6.0, for 50 minutes in a microwave oven. Endogenous peroxidase activity was blocked in 0.3% hydrogen peroxide in methanol. After a brief wash in Tris-buffered saline pH 7.6, tissue sections were incubated in MoAbs 1H4-1 and 2B4-1 for 1 hour at dilutions of 1:100 and 1:200, respectively. All antibodies were diluted in 10% normal sheep serum. Bound antibody was detected using rabbit antirat IgG (catalogue no. Z455; Dako Ltd, Glostrup, Denmark) at a dilution of 1:400, followed by biotinylated goat antirabbit (catalogue no. E432; Dako) at 1:100. Finally, the streptavidin ABC complex was applied (catalogue no. K492; Dako). Peroxidase activity was visualized by the standard diaminobenzidine reaction. Frozen sections were transferred directly to the citrate buffer step, and the immunostaining performed in the same way as for paraffin sections.

RESULTS

Biochemical characterization of MoAbs against EBNA1. MoAbs were generated using a bacterially expressed EBNA1-lambda-cro/lacZ fusion protein that is devoid of the "Gly-Gly-Ala" repeats of EBNA1.²⁹ MoAbs positive for EBNA1 reactivity in the solid-phase immunoassay were subsequently assayed by Western blot analysis for their ability to detect EBNA1 from a variety of cell lines. Clones that recognized additional (nonspecific) bands were not considered further. Clones designated EBNA1-1H4-1 (rat IgG2a) and EBNA1-2B4-1 (rat IgG1) were subcloned and analyzed in greater detail; these clones will subsequently be referred to as 1H4 and 2B4. A representative Western blot analysis using clone 1H4 is shown in Fig 1A. The right panel in Fig 1A shows the staining of EBNA1 expressed in baculovirus-infected insect cells; no signal was obtained with cell extracts derived from EBNA2A-expressing cells or cells infected with the wild-type (WT) baculovirus. In cell extracts from EBV-positive lymphoid cell lines, the 1H4 MoAb detected a single band corresponding to EBNA1, as verified by the different strain-specific electrophoretic mobilities of EBNA1 which are mainly because of differences in the length of the "Gly-Gly-Ala" repeats. The antibodies detected EBNA1 derived from a variety of LCL and BL cell lines and recognized EBNA1 from both type 1 and type 2 EBV isolates. It was noted that the EBNA2-deficient P3HR-1 cells expressed an increased amount of EBNA1 as compared with the parental, EBNA2B-expressing cell line Jijoye. The EBV-negative cell lines BL41 and BJAB did not yield a detectable signal.

Both 1H4 and 2B4 were also tested in immunoprecipitation analysis using extracts from different EBV-positive and EBV-negative cell lines. The result of an experiment using extracts of the EBV-positive B95.8 cell line and the EBV-negative BJAB cell line is shown in Fig 1B. 1H4 precipitated EBNA1 from the B95-8 extract, whereas, as expected, no signal was obtained with the BJAB cell extract cell line or with the irrelevant rat MoAb Rmt6. Both 1H4 and 2B4 de-

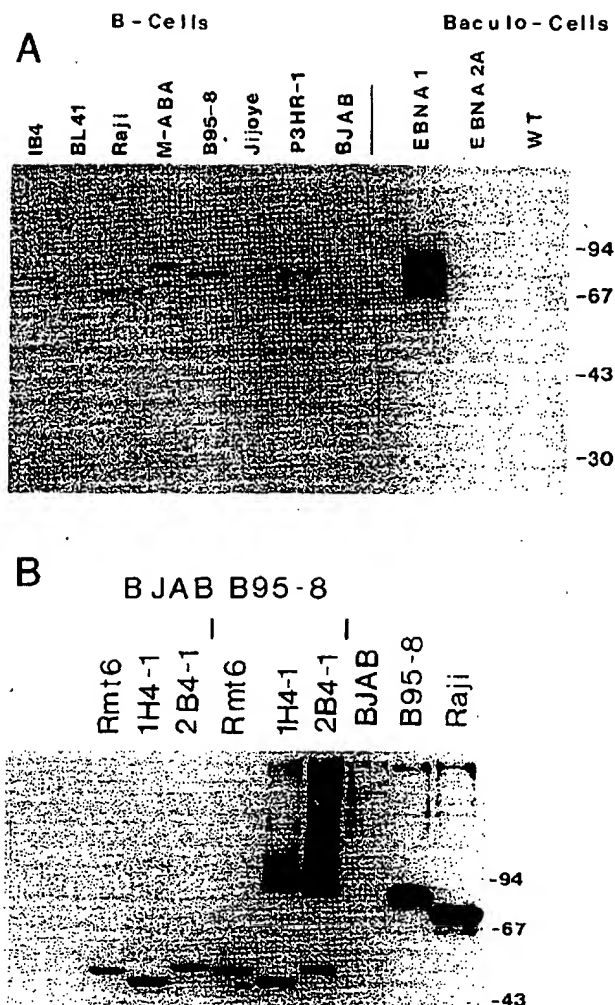


Fig 1. Specific detection of EBNA1 using the 1H4 MoAb. (A) Western blot analysis of various B-cell lines and of baculovirus-infected cells. The MoAb showed the characteristic size variation in the EBNA1 protein and did not react with EBV-negative B-cell lines (BL41, BJAB) or with SF158 insect cells infected with either EBNA2A or wild type (WT) recombinant baculoviruses. (B) Immunoprecipitation of EBNA1 from B95.8 cells (middle panel) or EBV-negative BJAB cells (left panel) using the two EBNA1-specific MoAbs (1H4 or 2B4) and a control rat MoAb (Rmt6). The immunoprecipitates were subjected to Western blot analysis using the 1H4 MoAb and the right-hand panel represents whole cell extracts run on the same gel. The reactivity at around 50 K in the immunoprecipitates is nonspecific, having been derived from Ig heavy chains present in the cell lines.

tected the EBNA1 protein only in a subset of cell lines by immunoprecipitation, reacting most strongly with the B95-8 EBNA1 used for immunization but also precipitating EBNA1 derived from the Raji or Jijoye BL lines. In immunofluorescence analysis, 1H4 and 2B4 yielded a clear signal in insect cells infected with EBNA1-expressing baculovirus, whereas almost no signal was obtained using EBV-positive B-lymphoid cell lines (data not shown). However, microwave treatment and the use of a second round of immunofluorescent staining resulted in the detection of EBNA1 in

both LCL and EBV-positive BL cell lines. Taken together, these data indicated that the antibodies reacted primarily with an epitope that became fully available only after denaturation.

Immunohistochemical detection of EBNA1 in established cell lines. In the next set of experiments, we attempted to show the presence of EBNA1 by immunohistochemistry in a variety of cell lines. A series of established LCLs (including X50-7, JY, and various B95.8-derived LCLs) as well as of type I (Akata, Wanyonyi, Rael, Odhi, Mutu c179, c59) and type III (Mutu c148, c62) BL cell lines were tested. EBV-negative cell lines tested included BJAB, BL2, Ramos, and the EBV-negative Hodgkin's line KMH2. Whereas the EBV-negative cell lines tested showed no reactivity with the MoAbs, strong nuclear staining with a pronounced granular appearance was observed for both 1H4 and 2B4 in all the EBV-positive lines tested (Fig 2A through D). This was observed in both cytospin preparations and paraffin wax sections of cell lines. No qualitative nor quantitative difference in the expression pattern between type I and type III BL cells was observed. Paraffin wax sections of B95-8 LCL-derived SCID tumors also showed strong labeling of tumor cells with both antibodies, whereas the EBV-negative Louckes-derived SCID tumors were not labeled.

Immunohistochemical detection of EBNA1 in clinical specimens. Finally, we analyzed paraffin wax sections from a series of EBV-associated lymphoid tumors. Of 31 EBER-positive HD specimens, 20 showed detectable expression of EBNA1 within HRS cells using the 2B4 MoAb, whereas 11 cases were positive with the 1H4 MoAb (Fig 2E and F). With one exception, all 1H4-positive cases were also 2B4-positive. However, in those cases in which EBNA1 could be detected, there was always a fraction of tumor cells that were not labeled. A total of 21 EBER-negative HD cases showed no labeling of HRS cells with either antibody. These negative cases were mostly of nodular sclerosis subtype (16 cases). In positive cases, the HRS cells showed a granular staining pattern that was similar to that observed in the cell lines, although in some cells the periphery of the nucleus was also strongly labeled. However, in general, the staining was weaker than that observed in the cell lines. The 2B4 antibody also appeared to react weakly with a subset of nonmalignant cells that included endothelial cells. This non-specific nuclear staining was readily distinguished from that observed in tumor cells. In EBER-positive cases, there was no relationship between EBNA1 expression and subtype of HD (Table 1). The immunohistological analysis was also extended to cryostat sections of HD. Of 9 EBER-positive specimens, 3 showed labeling of tumor cells with the 1H4 reagent, whereas 7 cases were positive with the 2B4 MoAb. The pattern of staining was identical to that observed in paraffin sections (data not shown). A total of 4 EBER-negative cases examined showed no labeling in tumor cells. Paraffin wax sections of BL and human immunodeficiency virus (HIV)-associated intracerebral B-cell lymphoma were also tested.³⁶ All 3 EBER-positive BL specimens and 8 of 8 HIV-associated lymphomas showed labeling of tumor cells with both MoAbs. Once again, the characteristic granular staining

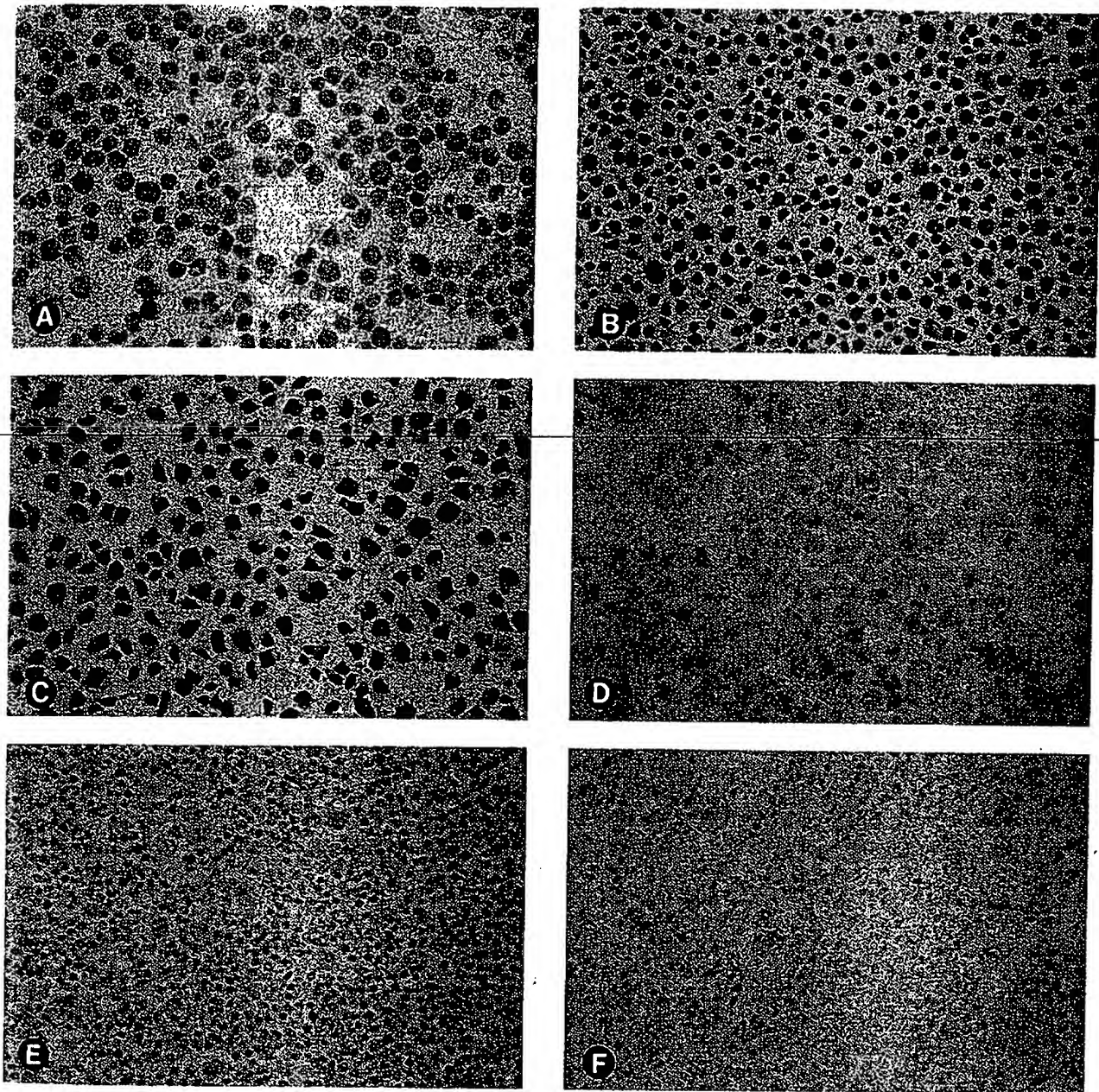


Fig 2. Immunohistochemical detection of EBNA1 in cell lines and clinical specimens. Using MoAb 2B4, EBNA1 expression is observed in most cells of (A) a lymphoblastoid cell line (B95.8), (B) a type I BL cell line (Mutu c179), and (C) a type III BL cell line (Mutu c148); whereas (D) an EBV-negative lymphoma cell line (BJAB) shows no reactivity. Nuclear EBNA1-specific staining of HRS cells is observed in two different cases of nodular sclerosis HD with MoAbs 1H4 (E) and 2B4 (F).

pattern was observed within tumor cells, but in most cases, the signals were much stronger than those observed in the tumor cells of HD (data not shown). A single EBER-positive T-cell lymphoma studied also showed labeling with both antibodies (data not shown). EBNA1 could not be detected in 8 EBER-negative large cell anaplastic lymphomas tested.

DISCUSSION

In this report, we describe the production and characterization of rat MoAbs directed against EBNA1. Two MoAb

clones (1H4 and 2B4) were chosen on the basis of their ability to specifically recognize EBNA1 in Western blot analysis of either baculovirus-expressed EBNA1 or a range of EBV-positive cell lines. By Western blot analysis the MoAbs detected EBNA1 in virtually all EBV-positive cell lines tested irrespective of the viral subtype. In contrast, these MoAbs reacted only poorly, if at all, in immunofluorescence analysis and were able to immunoprecipitate EBNA1 from only a subset of B cells. The ability of the MoAbs to readily detect EBNA1 by Western blot analysis

Table 1. Relationship Between HD Subtype and Detection of EBNA-1 in HRS Cells of EBER-Positive HD

Subtype	Frozen Sections		Paraffin Sections	
	IH4	2B4	IH4	2B4
Nodular sclerosis	3/6	5/6	4/11	6/11
Mixed cellularity	0/2	2/2	3/9	7/9
Lymphocyte predominant	0/1	0/1	1/3	2/3
Lymphocyte depletion	—	—	0/2	1/2
Unclassified HD	—	—	1/6	4/6
Total	3/9	7/9	9/31	20/31

while failing to reliably stain cells in immunofluorescence analysis suggests that the MoAbs recognize an epitope that is partially masked in the native protein but is exposed on denaturation.

Previous reports have shown that a proportion of HD cases contain EBV genomes and the EBV-encoded EBERs in the malignant HRS cells.¹²⁻¹⁴ Subsequently, the consistent expression of LMP1 in the HRS cells of EBV-positive HD was shown in the absence of EBNA2 expression.^{15,16} We recently analyzed EBV transcription in HD using reverse transcription polymerase chain reaction analysis and confirmed that the transcriptional program in HRS cells is similar to that observed in NPC.¹⁹ Of particular interest was the detection of selective EBNA1 expression from the Fp promoter that is also used to drive EBNA1 transcription in NPC biopsy specimens and BL cell lines which retain the original BL biopsy phenotype.^{20,21} Although this data strongly suggested that the EBV genome maintenance protein EBNA1 is expressed in HD, the paucity of HRS cells in HD biopsy specimens precluded direct demonstration of EBNA1 protein using the standard anticomplement immunofluorescence assay.³⁷ This assay is notoriously prone to non-specific cross-reactivities when applied to tumors and, because it is based on the use of selected EBNA-positive human sera and complement, is particularly problematic when used to analyze lymphoid malignancies. Furthermore, the presence of antibodies against the other EBNA proteins and lytic virus antigens in human serum can confound the interpretation of anticomplement immunofluorescence staining.

Thus, the aim of the present study was to develop MoAbs against EBNA1 and to show that EBV-positive HD, in particular, but also BL and HIV-associated B-cell lymphomas express EBNA1. The MoAbs were used to stain smears of B-cell lines or tissue sections of HD, BL, and HIV-associated cerebral B-cell lymphoma. The presence of EBNA1 could be shown in LCLs and BL cell lines using immunofluorescence or immunohistochemistry subsequent to paraformaldehyde fixation and microwave treatment of the smears. Similar treatment of paraffin-embedded or cryostat sections of HD, BL, and HIV-associated intracranial B lymphoma clearly showed the granular staining pattern characteristic of EBNA1 reactivity. To our knowledge, this is the first direct

demonstration the EBNA1 protein in tissue sections of these tumors.

In the established cell lines, most cells expressed detectable amounts of EBNA1, whereas in tissue sections variable numbers of tumor cells reacted with the EBNA1-specific MoAbs. It is possible that antigen availability and preservation may affect the detectability of EBNA1 and that this effect is not evident in tumors with a homogeneous population of EBV-positive cells. This explanation is supported by the observation that those tumors with the most intense EBNA1 staining tended to be those with the greatest number of EBNA1-positive cells. Furthermore, when extremely high levels of EBNA1 were expressed in the baculovirus system, the MoAbs were readily able to detect the protein without the need for microwave treatment. This suggests that the level of EBNA1 expression affects the detectability of the protein. Nevertheless, BL and HIV-associated B-cell lymphoma cells contained readily detectable amounts of protein, whereas HRS cells appeared to express only moderate or very low levels of EBNA1, and some cases of EBER-positive HD were negative for EBNA1. That this inability to consistently detect EBNA1 in EBV-positive HD is not caused by different subtypes of EBV or different transcriptional programs is evidenced by the ability to detect EBNA1 by Western blot analysis in all EBV-positive cell lines analyzed, including those carrying different EBV strains (ie, type 1 versus type 2 isolates)³⁸ and BL cell lines expressing EBNA1 from the Fp promoter (ie, group I versus group III).²⁵ Our overall conclusion is that, in a proportion of EBV-positive HD cases, a combination of low-level expression of EBNA1 and the problems of antigen preservation in paraffin-embedded material hampered the reliable detection of EBNA1 using immunohistochemistry.

The expression of EBNA1 in all forms of EBV latency is consistent with the essential role of this protein in viral DNA replication and maintenance of the viral episome.²² Studies in which specific antisense inhibition of EBNA1 resulted in the growth inhibition of a BL cell line emphasize the importance of EBNA1 in the development of BL.³⁹ Other data provide strong evidence that EBNA1 can participate in the deregulation of *c-myc* in the presence of a *myc/Ig* translocation through activation of the Ig enhancer.³⁹ Although this effect suggests an additional role for EBNA1 in the pathogenesis of BL, the transactivation of certain promoters by EBNA1 may also be relevant to the development of HD and other EBV-associated malignancies. Consistent with this hypothesis is recent data showing that transgenic mice expressing EBNA1 from Ig heavy chain enhancer develop lymphoma.⁴⁰ To date, every one of the EBV-latent proteins except EBNA1 has been shown to be a potential target for EBV-specific cytotoxic T lymphocytes.^{41,42} Therefore, it is tempting to speculate that the inability of EBNA1 to serve as a cytotoxic T lymphocyte target may facilitate its role in both the persistence and pathologic role of EBV. Although the site of EBV persistence in vivo remains unknown, on the basis of current evidence, it has been proposed that the B-lymphoid compartment is responsible for the maintenance of chronic EBV infection.² The availability of MoAbs

against EBNA1 that can be used in immunohistology will not only advance our understanding of the role of EBV in the various virus-associated tumors but will also be important in identifying the cell types and sites mediating EBV persistence.

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